IN VITRO ANTIBACTERIAL AND WOUND HEALING PROPERTIES OF A STANDARDIZED POLYPHENOLS-RICH FRACTION OF *DICRANOPTERIS LINEARIS* (BURM.) UNDERWOOD

By

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
α	Alpha
β	Beta
µ	Micro
δ	Chemical shift
λ_{max}	Maximum absorption
µmol TE/g	Micromol trolox equivalent
µmol GAE/g	Micromol gallic acid equivalent
µL	Microlitre
ACN	Acetonitrile
AMX	Amoxicillin
ANOVA	Analysis of variance
AR	Analytical grade
ATCC	American type culture collection
CFU	Colony forming unit
$CHCl_3$	Chloroform
cm	Centimeter
CO_2	Carbon dioxide
DMEM	Dulbecco`s modified eagle medium
DMSO	Dimethyl sulfoxide
DCM	Dichloromethane

dd	Doublet of doublet
DEPT	Distortionless enhancement by population transfer
D. linearis	*Dicranopteris linearis*
dq	Doublet of quadruplet
F5	Fraction 5
FICI	Fractional inhibitory concentration indices
FBS	Fetal bovine serum
FCR	Folin-Ciocalteu reagent
ESI	Electron spray ionization
EtOAc	Ethyl acetate
Fe^{2+}-TPTZ	Ferrous tripyridyltriazine
Fe^{3+}-TPTZ	Ferric tripyridyltriazine
FDA	Food and Drug Administration
FRAP	Ferric reducing antioxidant power
FT	Fourier Transform
g	Gram
H_2SO_4	Sulphuric acid
H_2O_2	Hydrogen peroxide
HMBC	Heteronuclear multiple bond coherence
HPLC	High Performance Liquid Chromatography
Hs27	Human foreskin fibroblast
HSQC	Heteronuclear Single Bond Coherence
IC_{50}	Concentration of a test substance required for 50% inhibition *in vitro*

INT	*para* iodonitrotetrazolium
KBr	Potassium Bromide
m	Multiplet
MBC	Minimum bactericidal concentration
MeOH	Methanol
mg/mL	Milligrams per milliliter
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate buffer saline
ppm	Parts per million
prep-TLC	Preparative thin layer chromatography
q	Quadruplet
R_f	Retention factor
rpm	Revolution per minute
s	Singlet
S.E.M	Standard error mean
TLC	Thin layer chromatography
UV	Ultraviolet
Vis	Visible

WHO	World Health Organization

LIST OF APPENDICES

SIFAT ANTIBAKTERIA DAN PENYEMBUHAN LUKA *IN VITRO* OLEH FRAKSI KAYA POLIFENOL TERPIAWAI *DICRANOPTERIS LINEARIS* (BURM.) UNDERWOOD

ABSTRAK

Dicranopteris linearis (Burm.) Underwood merupakan sejenis paku-pakis menjalar tanah yang digunakan secara tradisional untuk merawat pelbagai penyakit kulit seperti bisul, ulser dan luka luaran. Sehingga kini, tiada kajian saintifik yang dijalankan secara sistematik untuk menilai kegunaan etnofarmakologikal *D. linearis* untuk mempercepatkan penyembuhan luka.Dalam kajian ini, ekstrak MeOH daripada *D. linearis* telah diperkaya dengan sebatian bioaktif dengan menggunakan kaedah berpandukan bioaktiviti. Salah satu fraksi ekstrak (F5) yang diperoleh telah didapati memiliki aktiviti antioksidan dan aktiviti antibakteria yang signifikan terhadap *S. aureus* dan *P. aeruginosa*. Analisis telah dilanjutkan untuk mengenal pasti kompoun dalam fraksi aktif ini. Pemencilan sebatian telah dijalankan dengan menggunakan gabungan teknik kromatokrafi.gel silica fasa terbalik dan normal. Dua sebatian fenolik yang penting secara taksonomi *D. linearis*, iaitu dikotomain **A (2)** dan dikotomain **B(3)**, dua sebatian baru, 4-vinil fenol-1-*O*- (2'-*O*-*α*-ramnopiranosil) -*β*-glukopiranosida**(5)** dan 4-vinil fenol-1-*O*- (4'-*O*-*α*-ramnopiranosil)-*β*-glukopiranosida**(6)** bersama-sama dua lagi flavonoid yang telah dilaporkan sebelum ini, kaempferol 3-*O*- *β*-glukopiranosida**(1)** dan kaempferol 3-*O* -*β*-glukosil (1 → 2) -*β*-glukosida**(4)** telah dikenalpasti. Pencirian kompoun-kompoun tersebut telah

dijalankan dengan menggunakan pelbagai teknik spektroskopi seperti UV, IR, NMR dan MS. Untuk menentukan kandungan F5 dan pemiawaian fraksi induknya, satu kaedah HPLC-DAD yang baru telah dibangunkan dan disahsahihkan menggunakan sebatian yang telah dipencilkan sebagai penanda analisis. Kaedah ini didapati jitu dan persis dengan LOQ 1.56 µg/mL untuk sebatian 1, 4, 5 & 6 dan 15.6 µg/mL untuk kompoun 2. Kandungan sebatian telah didapati sebanyak 3.1% untuk sebatian 1, 3.4% untuk sebatian 2, 3.4% untuk sebatian 4, dan 26.8% untuk sebatian 5 & 6.Aktiviti antibakteria dan potensi penyembuhan luka fraksi terpiawai F5 *D. linearis* telah dikaji seterusnya dengan menggunakan beberapa cerakin *in vitro*. Daripada cerakin masa-membunuh bakteria, cerakin kepekatan minimum perencatan bakteria (MBC) dan imej mikroskopik elektron pengimbasan, fraksi F5 didapati memberi kesan bacteriostatik terhadap *S. aureus* dan *P. aeruginosa*. Tambahan pula, F5 dapat meningkatkan potensi aktiviti antibakteria beberapa antibiotik konvensional, iaitu, kloramfenikol, penisilin G dan ampisilin terhadap *S. aureus* dan MRSA. Sebaliknya, F5 didapati meningkatkan proliferasi sel-sel fibroblast dan mengaruh penghijrahan sel dalam cerakin calar luka. Tambahan pula, ia juga memberi kesan perlindungan sel-sel daripada tekanan oksidatif yang disebabkan oleh hidrogen peroksida dan tidak mengakibatkan kesan sitotoksik-ke arah kedua-dua sel fibroblast manusia dan tikus pada kepekatan yang diuji, 500µg/mL. Kesimpulannya, kajian ini memberikan maklumat baru untuk menyokong penggunaan tradisional tumbuhan ini untuk mengubati jangkitan kulit. Kajian *in vivo* perlu dijalankan untuk meneroka perkembangan potensi *D. linearis* sebagai agen penyembuhan luka.

IN VITRO ANTIBACTERIAL AND WOUND HEALING PROPERTIES OF A STANDARDIZED POLYPHENOLS-RICH FRACTION OF *DICRANOPTERIS LINEARIS* (BURM.) UNDERWOOD

ABSTRACT

*Dicranopteris linearis (Burm.)*Underwood is a ground creeping fern used traditionally to treat various skin related ailments, such as boils, ulcers and external wounds. To date, no scientific studies have been carried out to evaluate its ethnopharmacological use in wound infection in a systematic manner. In the present study, the MeOH extract of *D. linearis* was fractionated with the bioactive constituents following a bioactivity-guided approach. A fraction (F5) of the extract was obtained and it was found to possess significant antioxidant and antibacterial activity against *S. aureus* and *P. aeruginosa*. Analysis was further carried out to identify the chemical constituents of this active fraction. Isolation of the major constituents was carried out using a combination of reversed-phase and normal-phase silica gel chromatographic techniques. Two phenolic compounds which are taxonomically significant to *D. linearis*, namely dichotomain A **(2)** and dichotomain B **(3)**, and two new compounds, 4-vinyl phenol-1-*O*-(2'-*O*-α-L-rhamnopyranosyl)-*β*-D-glucopyranoside **(5)** and 4-vinyl phenol-1-*O*-(4'-*O*-α-L-rhamnopyranosyl)-*β*-D-glucopyranoside **(6)** together with two other known compounds kaempferol 3-*O*-*β*-D-glucopyranoside **(1)** and kaempferol 3-*O*-*β*-D-glucosyl (1→2)-*β*-glucoside **(4)** were obtained. Characterization of the constituents was carried out using various spectroscopic techniques such as UV, IR, NMR and MS. In order to determine the content of F5 and to standardize the fraction, a novel HPLC–DAD method was

developed and validated using the isolated compounds as analytical markers. This method was found to be accurate and precise, with an LOQ of 1.56 µg/mL for compounds 1, 4, 5& 6 and 15.6 µg/mL for compound 2. The contents of the marker compounds were found to be 3.1% for compound 1, 3.4% for compound 2, 3.4 % for compound 4, and 26.8 % for compound 5 & 6. Antibacterial and wound healing potential of the standardized F5 fraction of *D. linearis* was subsequently investigated using several *in vitro* assays. From the time killing assay, minimum inhibitory concentration (MIC) assay and scanning electron microscopic images, F5 was found to exert bacteriostatic effect against *S. aureus* and *P. aeruginosa*. Moreover, F5 was also able to potentiate the antibacterial effect of few conventional antibiotics, namely, chloramphenicol, penicillin G and ampicillin against *S. aureus* and MRSA. On the other hand, F5 was able to enhance the proliferation of fibroblast cells and induce cell migration in the scratch-wound assay. In addition, it protected the cells against hydrogen peroxide induced oxidative stress and was non-cytotoxic towards both human and mouse fibroblast cell lines up to the tested concentrations of 500µg/mL. Taken together, the present study provided novel information to support the traditional use of this plant for skin infections. *In vivo* studies are warranted to further explore the potential of *D. linearis* as a wound healing agent.

CHAPTER 1

BACKGROUND

Chronic wounds are a growing threat which affect a large portion of population and signify a considerable public healthcare burden worldwide. In developed countries such as United States and UK, the healthcare cost to treat non healing wound is estimated to be about 2.5-25 billion annually (Posnett & Franks, 2008;Sen et al., 2009). With the increasing incidences of post-surgical wound, diabetes and obesity worldwide, the burden of chronic wounds is expected to be on a rise. Wound healing is rarely seen as a problem in healthy individuals. Under normal circumstances, a wound usually follows through the reparative process in a timely and orderly manner. However, under certain physio-pathological conditions such as stress, obesity, diabetes, local infection, hypoxia, aging, malnutrition and immunodeficiency, the wound healing process may be impaired (Harding, 2008).

One of the critical factors that delay the wound healing process is bacterial infection. When the skin, which acts as the major epithelial barrier between the body and the hostile atmosphere, is wounded, it becomes vulnerable for microbial invasion. Among some of the common bacteria which cause wound infections are the Gram negative *P. aeruginosa* and Gram positive *S. aureus* (Guggenheim et al., 2009). Both *P. aeruginosa* and *S. aureus* are opportunistic bacteria that are found in soft tissues of the body and known to be harmless pathogens. However, in immunocompromised individuals such as AIDS patients and those suffering from chronic illnesses, infections caused by these bacteria may lead to serious problems. In the past, bacterial infection can be easily treated with antibiotics. However, with

the non-systemic and indiscriminate use of antibiotics over time, bacteria strains that are resistant towards these antibiotics began to develop leaving the conventional antibiotic ineffective. At present approximately 90–95% penicillin resistant *S. aureus* and 70–80% of methicillin resistant *S. aureus* strains have been reported around the world (Chambers, 2001; Casal et al., 2005). Hence, there is an urgent need for newer and more effective antibiotic agents. While the development of new antibacterial agent is costly and time consuming, an alternative strategy to address such problem is to use combination of drugs. Several studies have reported the synergistic effects of plant extract or natural product when it is used together with conventional antibiotics. For instance pomegranate extract, myricetin and pyridine alkaloid which were isolated from *Jatropha elliptica*, exhibited potent synergetic effect when they are employed together with conventional antibiotics such as chloramphenicol, gentamicin, ampicillin, tetracycline, and oxacillin (Braga et al., 2005; Lin et al., 2005). While conventional antibiotics are mostly of microbial origin, development of resistance towards these antibiotics are relatively easy due to random mutation compared to plant secondary metabolites which have vast and unique structures that are not of common to microbes (Cowan, 1999). Hence, recent studies have been focusing on combining conventional antibiotics with plant extract/compound in order to reduce the chances of developing antibiotics resistant bacterial strains (Ghaleb et al., 2010). This will not only prolong the effective lifespan of an antibiotic, it can also reduce the side effects caused by these antibiotics.

Dicranopteris linearis (local name: resam) is a fern used traditionally in Southeast Asia to control fever and to treat external wounds, ulcers, boils and intestinal worm infection (Piggott, 1988; Chin, 1992). Studies have shown that it

exhibits strong antioxidant effects and have antibacterial properties towards *B. cereus, M. luteus, S. aureus, P. aeruginosa* and *E. coli* (Lai et al., 2009). However, these few studies are insufficient to provide support for an evidence-based use of the plant for wound infection. As such the present work was carried out with the following specific objectives:

1. To prepare a fraction of *D. linearis* that with bioactive constituents
2. To carry out isolation, characterization and structure elucidation of major compounds found in the bioactive extract of *D. linearis*
3. To develop and validate a HPLC-DAD analytical method for standardization of the bioactive fraction of *D. linearis*
4. To evaluate the antibacterial properties and synergistic effect of the standardized fraction with conventional antibiotics
5. To evaluate the wound healing potential of the standardized fraction of *D. linearis* through *in vitro* models.

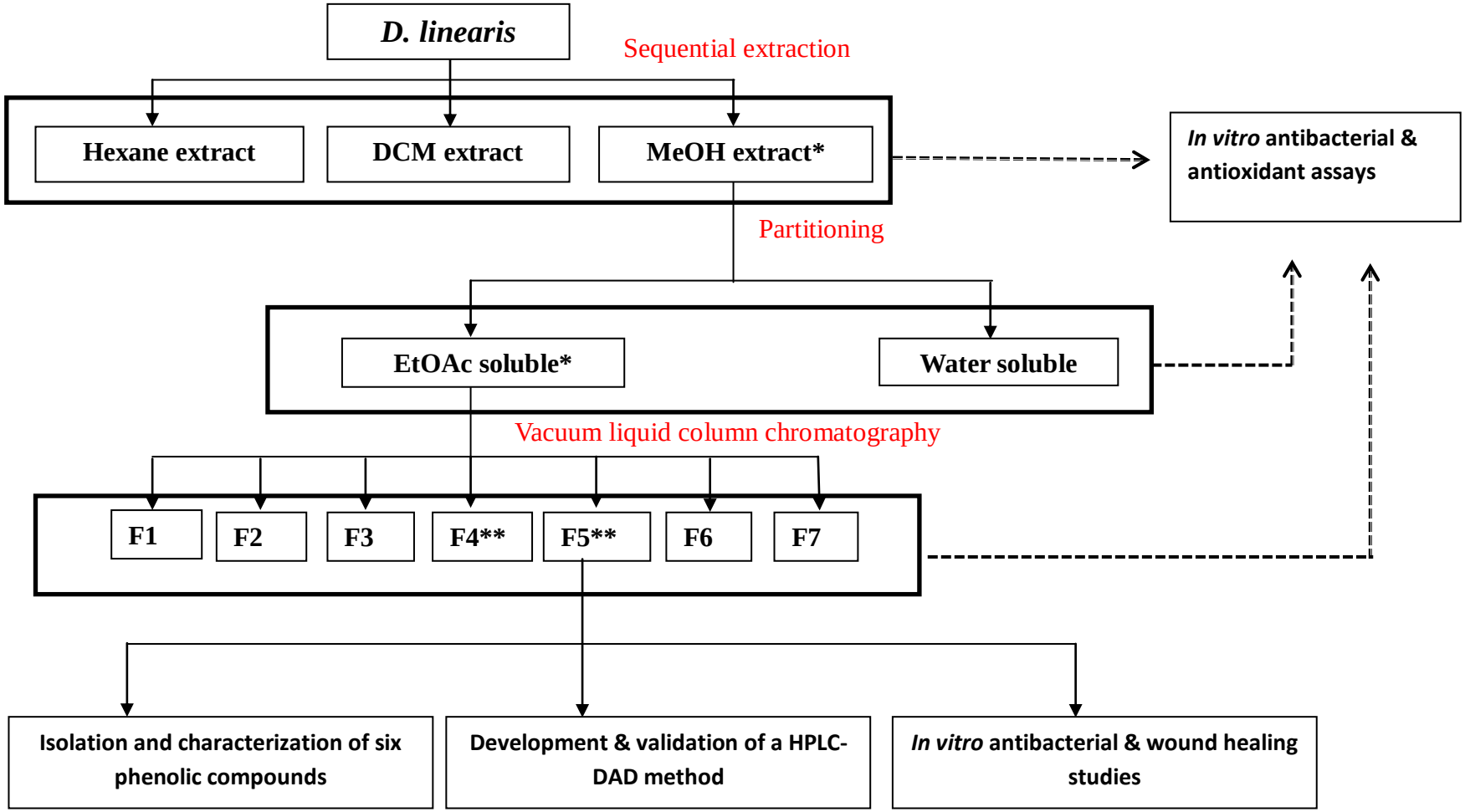


Figure 1.1: Research flowchart
* Denotes the active fraction

CHAPTER 2

LITERATURE REVIEW

2.1 Types of wound

In general, wound is referred to an injury of the skin, while infected wound is a condition in which microorganisms have colonized the wound causing either a delay in healing or deterioration of the wound. Infectious wounds vary in severity from benign to life threatening, mainly based on the virulence of the pathogen involved, the pathology of the affected area and the primary health condition of the patient (Fadeyibi et al., 2013). Skin damage can be classified into two major types, which is acute or chronic wound. External damage to intact skin such as surgical wounds, bites, burns, minor cut, abrasion and lacerations are classified as acute wounds (Bowler et al., 2001). Usually these types of wounds are expected to heal within a predictable time frame during which a series of healing processes occur naturally. Conversely, chronic wounds such as leg ulcers, foot ulcers, abscesses and pressure sores are most frequently caused by predisposed pathophysiological abnormalities of the patient. Health conditions such as diabetes mellitus which impair the tissue perfusion and other factors such as aging, obesity, smoking, poor nutrition, and immunosuppressive associated diseases may also aggravate the development of chronic wound resulting in slow and unpredictable healing (MacGregor, 2008). Although acute and chronic wounds differ in terms of their severity and pathological conditions, microbial infection remains as a common clinical problem to both types of wounds.

2.2 Bacterial activity in wound

Wound infection delays the healing process, which can eventually lead to other complications. Various bacteria causes wound infection and among them are the aerobic or facultative pathogens such as *S. aureus*, *P. aeruginosa*, and β-hemolytic *Streptococci* (Gadepalli et al., 2006). The emergence of antibiotic resistant virulent pathogens and poor disease management has practically led to a steady increase of infectious skin diseases. A study conducted by European Centre for Disease Prevention and Control (2009) estimated 380,000 healthcare associated infections annually in Europe Member States hospitals are due to antibiotic-resistant bacteria, including those of the respiratory tract, skin or soft tissues and urinary tract. Chambers and colleagues (2001) also reported that in most of the Asian countries, around 70-95% of *S. aureus* strains were found to be resistant to penicillin and methicillin. Besides, up to 75% of morbidity following injuries is also found to be related to infections (Church et al., 2006), usually caused by methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* (Vindenes & Bjerknes, 1995).

Staphylococci are non-motile, Gram-positive cocci that causes postoperative wound infection (Martone et al., 1992). In a healthy individual, more than 10^6 virulent staphylococci are needed to cause a minor infection, however in an immunocompromised person 10^2 is sufficient enough to initiate an infection. In addition, the number of organisms which are needed to initiate infection can also be reduced due to the placement of foreign medical devices such as central nervous catheters, hemodialysis catheters, tracheostomy tubes, intravascular catheters, artificial heart valves, sutures, and prosthetic joint devices (El-Ahdab et al., 2005). During a staphylococcal infection, asymptomatic carriers might persist to function as reservoirs for the organism probably due to formation of biofilm. Biofilms are

formed by adherent bacteria cells that can attach to living or non-living matrixes through a self-produced matrix of extracellular polymeric substance (EPS). Hence, the carrier state of staphylococcal infection cannot be easily eliminated by β-lactam antibiotics or vancomycin treatment. Various topical and systemic agents including rifampin, ciprofloxacin, minocycline, trimethoprim-sulfametoxazole, mupirocin, and novobiocin, have been used alone and also in combination for treatment of staphylococcus infections and the results are variable. Most of the time, the failure in treatment for such infections are due to the un-systemic use of antibiotic resulting in the emergence of resistant strains (Mulligan et al.,1993).

The rod shaped gram negative bacteria *P. aeruginosa* has been reported to be the second most frequent agent to cause skin infections (Hillier et al., 2008). Though in a healthy individual, *P. aeruginosa* rarely provokes infection, it poses serious health risk to immunocompromised patients. *P. aeruginosa* accounts for about 10% of infections among the in-hospital patients (Schimpff et al., 1970; Germiller et al., 2005). The ubiquitous nature of *P. aeruginosa* has made it very versatile, hence largely contributing to its ecological success as opportunistic pathogen. It can grow at a broad range of temperature (37-42°C) and it is also fairly resistant to disinfectants.

2.3 Antioxidant activity in wound healing

Antioxidants are molecules that can protect cells from oxidative damage by scavenging free radicals. At the wound site, as part of human`s innate immune system, there is tendency for a sharp rise in the concentration of reactive oxygen species (ROS) due to the stimulation of various inflammatory cells such as neutrophils, lymphocytes, macrophages and platelets (Waldorf & Fewkes, 1995).

Although small amount of these ROS are important to trigger off a cascade of healing events, excessive amount of these free radical are injurious to the surrounding tissues and can cause serious damage to it. To counter oxidative damage, body utilizes endogenous antioxidant enzymes such as superoxide dismutases, catalases, glutathione peroxidases and peroxiredoxins, secreted by the adjoining cells (Ghosh & Gaba, 2013).These compounds are reported to enhance cell proliferation and exhibit antibacterial effects. These properties are useful for the rapid healing while protecting the wounded site from deleterious effect of infection (García-Pérez et al., 2014).

2.4 Process of wound healing

The progression of wound healing can generally be divided into several stages such as coagulation, inflammation, cell proliferation and migration, re-epithelialization and subsequently tissue remodeling (Fitzmaurice et al., 2010), Figure 2.1. These phases of wound healing process are distinct yet overlapping. For an example, while events in inflammation phase are actively occurring, tissue formation process also begins. A unique group of cytokines regulate and synchronize wound healing. They are known as growth factors that are secreted from macrophages, neutrophils, endothelial cells, lymphocytes, fibroblasts and thrombocytes. Many of these cytokines play the role in wound healing processes, such as fibroblast-derived growth factor (FGF), tumour necrosis factor (TNF), platelet-derived growth factor (PDGF), granulocyte-macrophage colony stimulating factor (GM-CSF), epidermal growth factor (EGF), transforming growth factors (TGF) α and β, as well as insulin-like growth factor (IGF). When an open wound is generated, platelet plug and blood clot are formed to temporary seal the wound in a

process termed as coagulation. Platelet plug activates macrophages and other cells in the wound area to produce various chemokines, growth factors and cytokines, causing influx and activation of larger number of macrophages. The adaptive immune system response is then stimulated (Waldorf & Fewkes, 1995). Inflammatory phase which usually lasts for about 4 – 6 days in normal course of wound repair is then initiated where a number of antibodies, immune cells, enzymes and growth factors are recruited to the wound area. Reactive oxygen species (ROS) are also released in large amount to protect the wound against invading microorganism (Schafer & Werner, 2008). Taking cue from the inflammation phase, the most prominent event of wound healing process, the cell proliferation and migration phase are initiated. During proliferation phase, the fibroblast cells replicates and angiogenesis of new capillaries also occurs to support the development of granulation tissue (Ito et al., 2007). The newly formed capillaries branch out and invade fibrin matrix at the wound site, whereby it forms a branched and complex vascular network. Proliferation of certain cell lines such as epithelial cells, fibroblasts, and endothelial cells was reported to be induced by various growth factors (Kim et al., 1998). After the proliferation phase, epithelialization process then takes place where epithelial cells resurface and fills up the wound gap. The migration of epidermal cells can usually be seen from the margins of the wound to the wound matrix (Kanzler et al., 1986). Finally, the wound healing progresses into the remodeling phase, during which the wound strength increases as the fibroblasts organize and cross-link the collagen. The wound then contracts and the capillary and fibroblast density regress and decrease (Hsu & Mustoe, 2010).

Since wound healing is a highly organized process, an active wound healing agent should contribute in one or more phases of the healing process, in a proper

sequence and at the right time frame to show improved efficacy. Preferably a plant-based remedy should promote at least two different processes before it can be said to have some scientific support of its traditional use for wound healing (Houghton et al., 2005). It should exhibit three main effects; antioxidant, antimicrobial and cell proliferation activity in order to contribute positively to the wound healing process (Ghosh & Gaba, 2013).

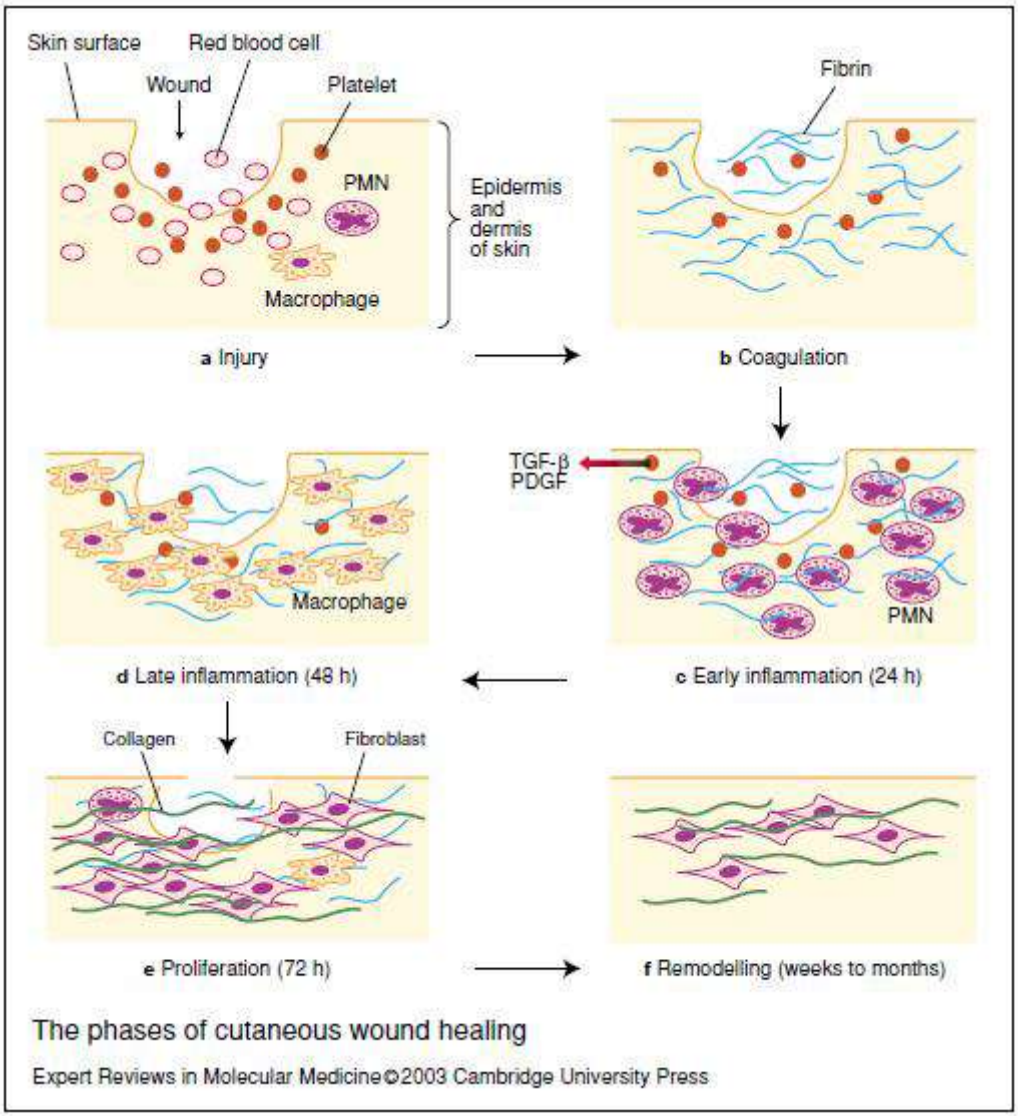


Figure 2.1: Phases of cutaneous wound healing (Patrick et al., 2013)

2.5 Plant based medicine for wound healing

Herbal medicines have been used since the ancient civilisation for the healing of wounds. Even today with the advancement attained in modern medicine, many people are still relying on herbal medicines as an alternative therapy to heal wound due to the cost effectiveness, accessibility and lesser side effects of herbal preparations. During the last two decades, there have been increased interests to utilize plant preparations in wound healing and to understand the active constituents that promote and modulate the healing process. There are numerous scientific reports on plant such as aloe vera, turmeric, lavender and tea tree that are used traditionally to accelerate wound healing. In many cases the scientific findings were found to substantiate the traditional use of the respective medicinal plants (Krishnan, 2006). Selection of plants for these scientific studies are often guided by traditional practices such as Ayruvedic medicine, Traditional Chinese Medicine (TCM) and folk medicine (Kumara et al., 2007; Rawat et al., 2012;Wang et al., 2013). For instance, turmeric, the yellow powder used as spice, is usually applied as paste at the wounded area and the juice is taken orally to reduce inflammation (Aggarwal & Prasad, 2011). These traditional uses prompted systematic scientific research to learn more about the active constituents, the pharmacological properties and the absorption nature of this herb. Studies were carried out at pre-clinical and clinical stages and turmeric was found to be a promising candidate to be developed into a wound healing agent (Krausz et al., 2015). This is a typical example on how traditional practices of using plants provide pharmacological basis for their development into wound healing therapeutics.

2.6 Ferns with wound healing and antibacterial properties

Malaysia is one of the countries where Pteridophytes are found in abundance. Ferns belong to Pteridophyta, are known for its medicinal values and it had been widely used in the traditional medicinal system. Two species from the genus Davallia, *Davallia mariesii* and *Davallia solida* have been used as painkiller, anti-inflammatory agents and also for healing of fractured bones (Chang et al. 2007; Whistler, 1992). *Dynaria fortunei* and *Microsorum scolopendria* belongs to the same family have been used for skin inflammation, lumbago treatment and wound healing (Lee et al., 2008; Bloomfield, 2002). *Acrostichum aureum* is used to treat wound and ulcer (Morrison et al.,1994) while *Blechnum orientale* are being used as tonic and to cure wound cicatrization (Defilipps et al., 1998). The rhizome extract of *Drynaria quercifolia* was found to inhibit the growth of at least 6 bacteria strains significantly (Kandhasamy, 2008). Phytochemical studies revealed that Pteridophytes are rich in triterpenoids, sesquiterpenes, catechins, flavonoids, steroids and glycosides which are important source of therapeutic drugs (Anuja et al., 2010; Liu et al., 1999; Economides & Adam, 1998). Most of the reported phytochemicals are known for their radical scavenging activity.

2.7 Commercial products of fern with wound healing properties

One of the products developed from a medicinal fern used traditionally in South America to treat dermatitis is “Anapsos”. The product is registered under Spanish Health Department and it contains the standardized extract of a fern, *Polypodium leucotomas* as the active ingredient. The product is widely used to treat dermatological disorders such as psoriasis and atopic dermatitis and it is also found to be effective as photoprotectant, immunomodulator and antioxidant (Caccialanzaet

al., 2007).*P. leucotomas*, has also been used to develop another skin product called "Fernblock" which is used as an anti-aging and sun-block cream. Several major compounds such as 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillin acid, caffeic acid, 4-hydroxy cinnamic acid, ferulic acid and five chlorogenic acid isomers had been identified from the *P. leucotomas* extract and they are used to standardize the product (Carcia et al., 2006).The success of these products indicates the potential use of some ferns to be developed into pharmaceutical products to treat skin infections.

In spite of the traditional uses of a number of ferns for treating skin related ailments, the potential use of them as novel source of drugs for the treatment of skin diseases is still poorly explored. Only a small number of these plants have been investigated phytochemically and to a lesser extend studied systematically for their pharmacological properties. One such fern that had been used traditionally to treat skin diseases, but has rarely been investigated for its pharmacological action is *D. linearis* (Burm.) Underwood.

2.8 *Dicranopteris linearis* (Burm.) Underwood

2.8.1 Taxonomy and distribution

Dicranopteris linearis (Burm.) Underwood (synonym: *Dicranopteris dichotoma*,(Iwatsuki et al., 1993) also known as "resam" is a creeping fern indigenous to Malaysia. It belongs to the Gleicheniaceae family and subfamily of Gleichenioideae. This plant grows horizontally at ground level with a typical two main dichotomous forking stem which also branch further to about 3–4 times. Usually, the sporangia, where spores are produced and stored, are found under the mature fronds of *D. linearis*. The rhizome is several meters long and grows up to

5mm in diameter. The plants rapidly grow into thickets up to two meters tall. An illustration of *D. linearis* (Burm.) Underwood is given in Fig 2.2.

D. linearis is widely distributed in the subtropical and tropical regions including Southeast Asia, Polynesia, Central Africa, Madagascar and Northern & Southern America (Zhao et al., 2012), Figure 2.3. There are four common *Dicranopteris* species, namely, *D . linearis, D. pectinata*, *D. flexuosa* and *D. curranii* which are found in humid subtropical and tropical regions of the world. Among them *D. curranii* and *D. linearis* are found to be predominant in Malaysia (Negishi et al., 2006). *Dicranopteris* species are well adapted to highly-lit area; it grows and expands fast under this condition (Kochummen, 1977; Russell et al., 1998).

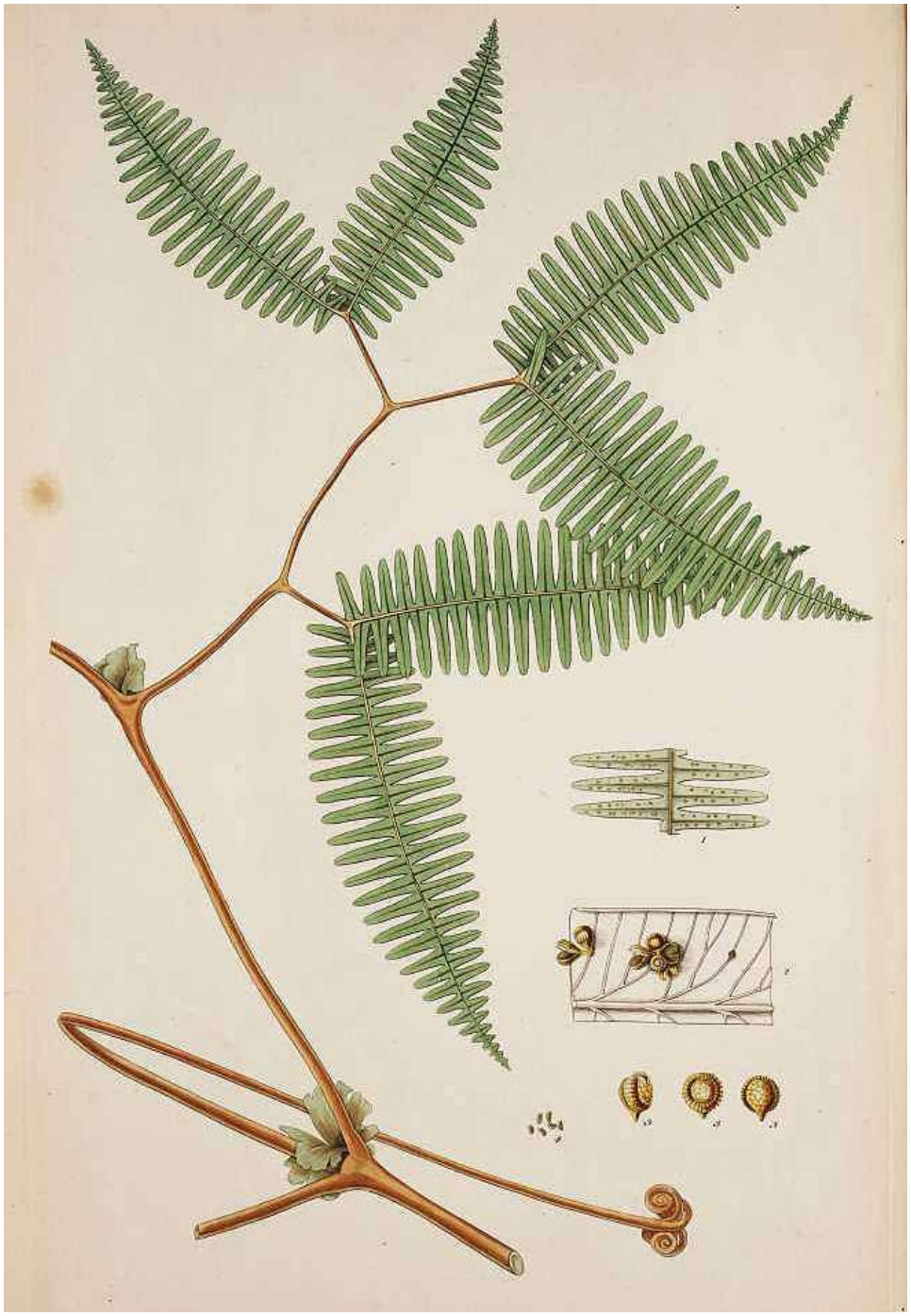


Figure 2.2: *Dicranopteris linearis* (Burm.) Underwood.

Source, Hooker & Greville, 1831

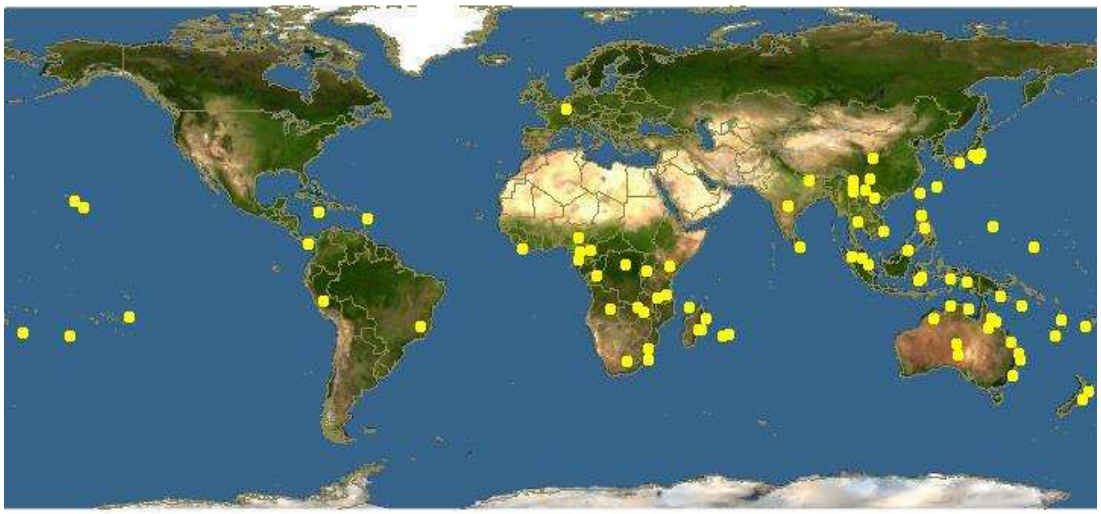


Figure 2.3: Geographical distribution of *D. linearis*, the yellow squares indicate the presence of *D. linearis*
(Source: http://www.discoverlife.org/mp/20q?search=Dicranopteris+linearis)

2.8.2 Ethnopharmacological relevance of *D. linearis*

Traditionally the leaves of *D. linearis* are crushed and made into poultice, to treat fever, as wound dressing and as an anthelminthic (Upreti et al., 2009). The decoction is drunk in Thailand against insomnia and to wash children with skin rash while in Hawaii it is used for constipation (Chin, 1998). In Malaysia, *D. linearis* is used to treat some of the chronic wounds like, ulcers, abscesses, boils, sores and gastritis (Piggott, 1988; Chin, 1998).

2.8.3 Research background of *D. linearis*

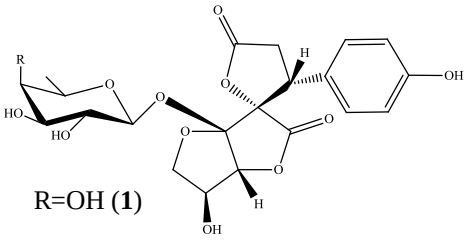
Several studies showed that the crude extracts of *D. linearis* possess antioxidant (Lai et al., 2009), antibacterial (Liu et al., 1999), anticancer (Rodzi et al., 2013) anti-inflammatory (Zakaria et al., 2011) and gastro-protective effect (Hussaini et al., 2012). The methanol and chloroform crude extracts of *D. linearis* was reported

to exhibit hepatoprotective effect which could be due to the presence of antioxidant compounds such as rutin and quercetin (Mamat et al., 2013; Ismail et al., 2014; Kamisan et al., 2014). In addition, the water and chloroform extract of *D. linearis* was found to possess antinociceptive and antipyretic activities (Zakaria et al., 2006; Zakaria et al., 2008).

2.8.4 Phytochemical constituents of *D. linearis*

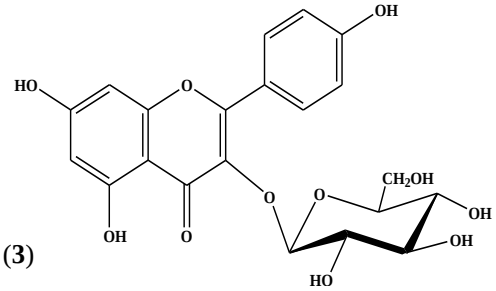
The phytochemicals of *D. linearis* have not been extensively explored. Only a handful of publications can be found regarding the identification of natural products from this species. Two main classes of compounds which have been reported from *D. linearis* are the polyphenols and terpenoids. Among the polyphenol compounds reported, the two highly oxygenated phenolic compounds with a spiral dilactone structure, dichotomain A (**1**) and dichotomain B (**2**) are found to be exclusive to *D. linearis* (Li, et al., 2006). Hence they are taxonomically significant to this species. Astragalin (**3**), quercetin (**4**) and rutin (**5**) are some of the common flavonoids isolated from *D. linearis.* Li et al., 2007 isolated few tetranorclerodanes and clerodane-type diterpene glycosides from the acetone extract of *D. dichotoma,* which is the synonym of *D. linearis.* Two clerodane-type diterpene glycosides, namely (6-*O*-acetyl-*β*-D-glucopyranosyl-(1→4)-R-α-rhamnopyranosyl) cleroda-3,14-dien-13-ol (**6)** and (4-*O*-acetyl-*β*-D-glucopyranosyl-(1→4)-R-α-rhamnopyranosyl) cleroda-3,14-dien-13-ol **(7**), 18-hydroxyaylthonic acid (**8**), 18-oxo-aylthonic acid (**9**), aylthonic acid (**10**) and cleroda-3, 14-diene-6, 13-diol (**11**) are some of the compounds that were isolated and identified by Li and his colleagues (2007). The same research group also isolated and reported few other terpenoids from *D. linearis*, clerodane-

type diterpene glycoside **(12),** 15-hydroxylabd-8(17)-en-19-oic acid **(13)** and junicedric acid **(14)** (Li, et al., 2008).

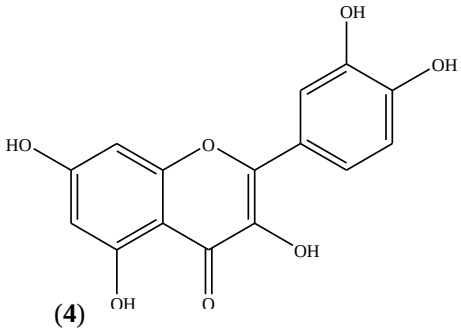


R=OH (**1**)

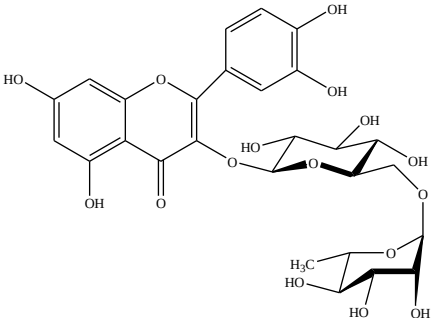
R=OCH_3 (**2**)



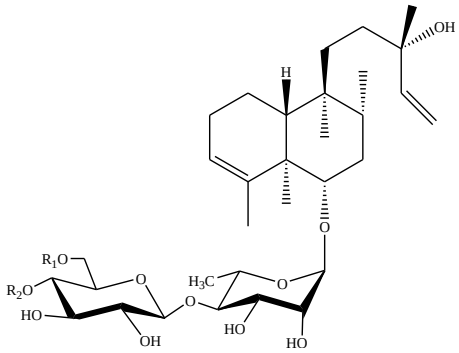
(**3**)



(**4**)

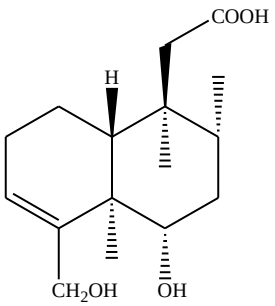


(**5**)

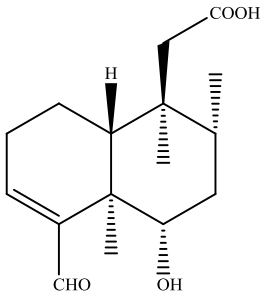


(**6**)=R_1=Ac, R_2=H

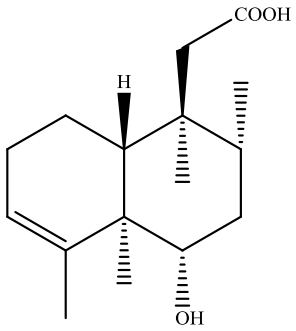
(**7**)=R_1=H, R_2=Ac



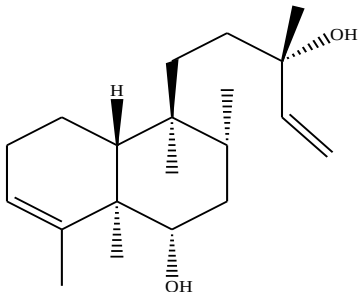
(**8**)



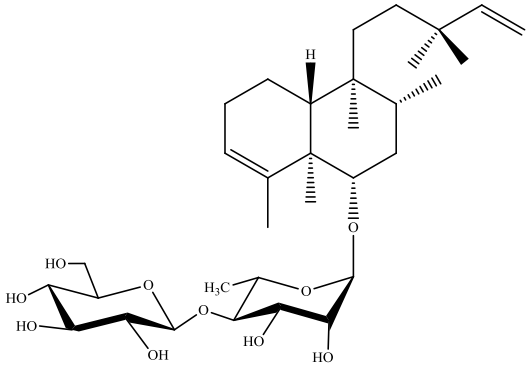
(**9**)



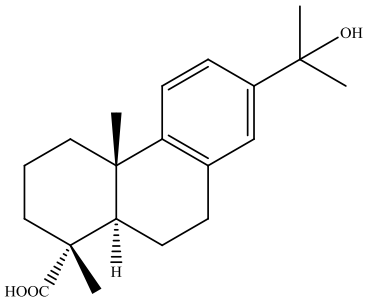
(**10**)



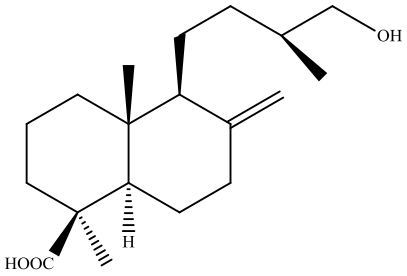
(**11**)



(**12**)



(**13**)



(**14**)

Figure 2.4: Chemical structures of compounds isolated from *D. linearis*

2.9 Isolated compounds versus herbal drugs

Plant-derived products have dominated the human pharmacopoeia for thousands of years and remained as an endless source of medicine. The discovery of

aspirin, a synthetic drug derived from a natural product salicylic acid in 1897 however had shifted the herbal medicine paradigm to mono-drug therapeutics. Mono-drug therapeutic concept involves the use of a single plant derived or synthetic drug to treat complex diseases. The concept of identifying a single chemical compound responsible for the therapeutic effect of a plant has thus been the paradigm shift in drug discovery in natural product (Williamson, 2001). With the advent of structure activity-guided organic synthesis and high throughput screening (HTS), this approach is easier to realize compared to using the conventional screening approach. The use of single compound provides the ease of studying the pharmacokinetics and pharmacodynamic properties, the mechanism of actions, and the side effects (Mohammad et al., 2005). There are numerous examples of drugs originated from natural products. Quinine, penicillin and pilocarpine are some of the earliest drugs derived primarily from natural sources (Daniel et al., 2012). Morphine and digoxin are also examples of well-established drugs. Morphine was isolated from opium produced from the poppy plant (*Papaver somniferum*) while digoxin is a heart stimulant originated from the flower of *Digitalis lanata* (Ramesh & Sudhir, *2015).* Some newer drugs such as artemisinin (antimalarial), paclitaxel and irinotecan (anticancer) are also derivatives of natural products (Harvey, 2008; Katiyar, et al., 2012).

Contradictory to the mono-therapeutic approach is the use of plant extracts or herbal preparations which consist of a mixture of varied compounds. These preparations may be obtained from a single plant or a mixture of various plants. The use of herbal preparations has formed the basis of treatment in most traditional medicine systems. The aim of using multicomponent herbal preparations is to attenuate certain medical properties, reduce toxicity and to improve bioavailability of

the pharmacologically active principles (Schmidt et al., 2008). Most frequently, the therapeutic effect of plants are due to the combinational effect of several compounds and in many cases the potency of the whole plant declines as it is purified into fractions or single compounds (Cravotto et al., 2010). Repeated fractionation and isolation process have a bottleneck effect to the complex plant extract, leading to the loss or reduction of the suboptimal active compounds which may have other pharmacological properties (Raskin & Ripoll, 2004). It is interesting to note that, with the rising occurrence of resistance and variable response to treatments, researchers are reverting to the "synergistic" approach, where combinations of different drugs with different targets are recruited. As the plant extract contains pool of compounds and it is cheaper to produce, this approach has a greater advantage over the use of single drugs. Enhanced bioavailability, synergy, and the additive properties of the constituents are the obvious advantages of the herbal medicine.

2.10 Standardized plant extract for pharmacological studies

In many parts of the world, plant derived medication still forms an integral part of healthcare and are being used to treat myriad of diseases. However many herbal preparations do not have a defined dosage, information on their chemical compositions and possible adverse reactions. Further to this is the poor quality control of these herbal preparations resulting in inconsistent and variable pharmacological effects (Canuto et al., 2012). Therefore, standardization of the plant extracts and herbal preparation have become an important measure for application in clinical and pharmacological practice (Eng, 2004). Standardization of plant extracts requires complete quality control at all stages, from the raw material to finished product (Carmona & Pereira, 2013). It involves the collection of comprehensive data

about the source of plant material and the fingerprint of the extract with at least four marker compounds (Garg et al., 2012). The information about the plant material such as appearance, maturity, drying and storage conditions can be collected through careful observation on the growing and extraction techniques while the fingerprint of the extract can be obtained through various analytical techniques such as TLC, HPTLC, HPLC and GC. Standardization is important to ensure the reproducibility and effectiveness of the herbal preparations extract while ensuring the chemical consistency, reproducibility of the pharmacological effect and the therapeutic efficiency (Bauer, 1998).

2.11 Separation techniques

In order to standardize the plant extract or fraction, identification of the bioactive or marker compounds is crucial. Various separation techniques can be used to separate and isolate the constituents according to its chemical nature. In most separation methods, the principle separation takes place through the selective distribution of the compounds between the mobile phase and the stationary phase.

Thin layer chromatography (TLC) is one of the most frequently used, cost effective, quick separation methods that have employed since many decades ago (Irena et al., 2009). The adsorbents such as silica or reverse phase silica (e.g.: C_{18}, C_8, C_4, C-cyclohexyl, C-phenyl and C-cyano) coated on aluminum or glass plates are used for TLC. TLC plates are usually supplied in 20cm × 20cm size and it can be cut into various sizes according to the user's preference. The mixture of natural products is spotted at the origin of the TLC plate and developed in a glass jar with saturated atmosphere of the developing solvent. The solvent is varied by mixing two or more solvents with different polarity. The separation of the mixture at the sample origin

occurs according to the polarity of the mobile phase of as it moves up on the TLC plate due to capillary action. The basic information of the chemical nature of the compounds is obtained by viewing the TLC plate under ultraviolet (UV) illumination or by spraying with appropriate reagent. TLC is a preliminary step to identify the optimal solvent combinations for further identification and purification of the mixture (Gilbert & Martin, 1998).

Once the optimal separation has been attained on TLC, the same developing solvent system can be applied on preparative thin layer chromatography (Prep-TLC). Prep-TLC is a scale up procedure of TLC which is used to separate and isolate the mixture at a larger scale. However, most frequently this technique is used as a final step of purification when the mixture contains less than five compounds in order to isolate the pure compounds. The separated compounds are detected by non-destructive UV method or by reacting with a reagent. The particular separated bands are then scraped out from the plate and desorbed using an appropriate solvent that is able to elute the compound.

Another inevitable separation method in natural products research is column chromatography. Two classical types of column chromatography are the open column and flash column. Open column uses the gravitational force to elute out separated compounds, while flash column chromatography uses external air forces such as nitrogen pressure, vacuum suction or control pump for compound elution. Though open column is more cost effective, recently it has largely been replaced by flash column chromatography methods due to shorter eluting time, efficiency and better separation. In column chromatography the selection of appropriate mobile phase composition, diameter and length of the column, as well as the sample loading size are essential to purify individual chemical compounds from a mixture of

compounds. The selection of these chromatography parameters depends upon the complexity of the sample mixture and the types of interactions between the mixture and the stationary phase (Ren et al., 2013).

Dry column vacuum chromatography (DCVC) is an alternative to flash column chromatography. Generally it uses less solvent and reduces the required amount of silica compared to a wet column. Unlike flash column chromatography where the sorbent bed is always maintained wet, DCVC uses a fractionation procedure in which after each step of gradient elution the column will be dried out before the addition of the next solvent system. This method is usually used for separating highly complex crude mixture into smaller fractions (Pedersen & Rosenbohm, 2001). In addition to these classical chromatographic techniques, there are also other more sophisticated, high-end separation technologies nowadays, such as high performance liquid chromatography (HPLC), counter current chromatography and supercritical fluid chromatography which provides more specificity and defined control over compound separation through their automated systems. By combining various chromatographic techniques at various stages of the purification process, reasonably pure compounds can finally be obtained from the crude extracts.

2.12 Compound characterization and structure elucidation

There are several spectroscopy and spectrometry techniques available to elucidate the structure of the isolated compounds. Spectroscopy is the means of understanding the interaction between matter and radiated energy while spectrometry is the method used to obtain a quantitative measurement of the spectrum.

Ultraviolet-visible (UV-Vis) spectroscopy is based on the electronic transition of the chemical compounds which absorb light in the UV-Vis region.